EXHIBIT A

Experimental

Hypoxia and VEGF Up-Regulate BMP-2 mRNA and Protein Expression in Microvascular Endothelial Cells: Implications for Fracture Healing

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The endothelium is a metabolically active secretory tissue, capable of responding to a wide array of environmental stimuli. Hypoxia and vascular endothelial growth factor (VEGF) are two components of the putative fracture microenvironment. This study investigated the role of hypoxia and VEGF on endothelial cell activation as it relates to the bone repair process. It was hypothesized that endothelial cells may have an important osteogenic role in fracture healing through the production of bone morphogenetic protein-2 (BMP-2), an osteogenic cytokine at the fracture site. Therefore, BMP-2 mRNA and protein expression in endothelial cells under hypoxia and/or VEGF treatment was studied. The authors observed a 2-fold to 3-fold up-regulation of BMP-2 mRNA expression in bovine capillary endothelial cells and human microvascular endothelial cells stimulated with hypoxia or rhVEGF. Furthermore, the combined effects of hypoxia and rhVEGF appeared to be additive on BMP-2 mRNA expression in bovine capillary endothelial cells. Actinomycin D and cycloheximide studies suggested that the increased mRNA expression was transcriptionally regulated. BMP-2 protein expression was up-regulated after 24 and 48 hours of treatment with either hypoxia or rhVEGF in bovine capillary endothelial cells. Surprisingly, the data suggest that endothelial cells may play not only an angiogenic role but also an osteogenic role by a direct stimulation of the osteoblasts, through the enhanced expression of a potent osteogenic factor, BMP-2, at the fracture site. (*Plast. Reconstr. Surg.* 109: 2384, 2002.)

Because of their strategic location between blood and tissues, endothelial cells are able to interact with numerous cell types. In addition to its primary function as an antithrombotic surface and a barrier for blood constituents, the endothelium is now considered to be a metabolically active secretory tissue.[1–4] Endothelial cells are able to respond to a diverse array of extracellular stimuli, expressing an altered phenotype referred to as "endothelial cell activation."[4,5]

During the first few days after a fracture, the callus microenvironment is relatively anaerobic.[6] Indeed, vascular disruption, together with a cascade of inflammatory events including enzymatic release from necrotic tissues and α-adrenergic–mediated vasoconstriction, creates a hypoxic gradient in which fracture healing initially begins.[6,7] Because hypoxia is present at the fracture site, we investigated the effects of reduced oxygen tension on endothelial cell activation as it might apply to bone repair and remodeling.

The metabolic and molecular changes occurring in endothelial cells exposed to hypoxia have been well studied.[8–10] For example, endothelial cells respond to hypoxic stress differently, depending on the duration of hypoxia. Acute hypoxia results in a quick and adaptive endothelial response, whereas chronic hypoxia leads to irreversible remodeling of the vasculature and surrounding tissues.[5,9] Furthermore, chronic hypoxia has been shown to directly regulate the expression of several genes in endothelial cells. For example, chronic hypoxia up-regulates endothelial cell expression of mitogenic factors such as transforming growth factor-β1 (TGF-β1), platelet-derived growth factor-BB (PDFG-BB), endothelin-1 (ET-1), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), thrombospondin-1, and others.[2,8,9,11–13] In addition, chronic hypoxia is known to down-regulate endothelial expression of antimitogenic

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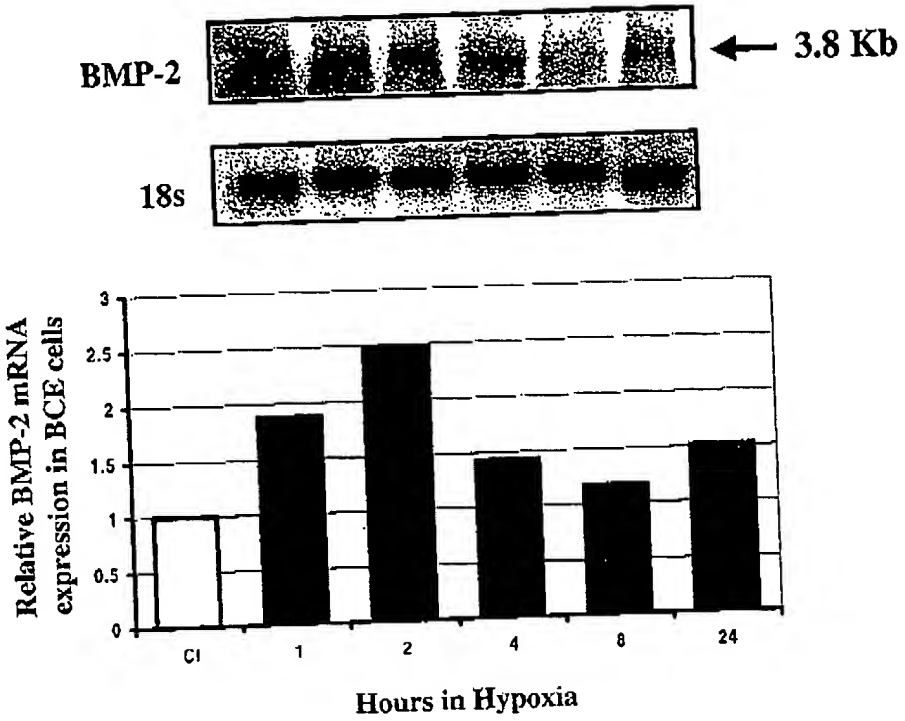

FIG. 1. The effects of hypoxia on BMP-2 mRNA expression in bovine capillary endothelial cells in vitro. Confluent bovine capillary endothelial cell cultures were placed in hypoxia for 1, 2, 4, 8, or 24 hours or normoxia (*Cl*, control). A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression at the indicated time points. The intensity of BMP-2 hybridization is given as a value relative to unstimulated bovine capillary endothelial cells. Hypoxic conditions resulted in a 2.5-fold increase in BMP-2 mRNA expression approximately 2 hours after exposure to hypoxia. BMP-2 mRNA expression then gradually returned toward baseline levels. This experiment was performed in duplicate.

factors such as endothelial nitric oxide synthetase and prostacyclin.[5,9] Hence, cell proliferation and matrix synthesis in tissues likely reflect a balance between these proliferative and antiproliferative stimuli regulated by oxygen tension through the endothelium. Furthermore, in the case of chronic hypoxia, the balance is clearly shifted to promote proliferation and matrix synthesis. This permanent structural remodeling is a key etiologic factor in pathologic conditions such as pulmonary hypertension or chronic venous insufficiency.[3,9,14]

Among the different cytokines involved in fracture repair, bone morphogenetic protein-2 (BMP-2) is one of the most studied. BMP-2 is a member of the TGF-β superfamily of cytokines that are key regulators of bone development, induction, and repair.[15–17] The bone morphogenetic proteins are a family of secreted growth factors that regulate a myriad of cellular processes on the basis of their extracellular concentration gradient.[18–20] The in vitro and in vivo effects of BMP-2 on osteoblasts have been well characterized, and BMP-2 is now considered to be a very potent osteogenic cytokine involved in fracture repair and bone formation.[15,17,21] For example, BMP-2 has been shown to be transcriptionally up-regulated in the osseous regenerate during endochondral distraction osteogenesis.[22]

VEGF is also involved in the fracture repair process. It is a secreted protein primarily involved in angiogenesis.[23] Recently, it has been linked to the bone remodeling process. For example, VEGF is required for cyclical blood vessel proliferation in longitudinal bone growth and endochondral bone formation.[23,24] In addition, osteoblasts produce VEGF in response to factors found at the fracture site, such as hypoxia, TGF-β1, FGF-2, and others.[25–29] Therefore, VEGF is now considered to be a key mediator in the capillary invasion required during bone formation.[24,30]

In this study, we attempted to determine whether endothelial cells could have a direct effect on bone remodeling through the expres-

sion of an osteogenic cytokine, BMP-2, at the fracture site under hypoxic conditions and/or VEGF stimulation. In an effort to answer this question, we chose to study BMP-2 mRNA and protein expression in two types of microvascular endothelial cells (bovine capillary and human microvascular endothelial cells) after stimulation with hypoxia and/or VEGF.

Materials and Methods

Cells and Media

Bovine adrenal capillary endothelial cells were a gift from Dr. G. Seghezzi (New York, N.Y.).[31] Cells were grown to confluence in α-modified minimum essential medium (Fisher Scientific Co., Pittsburgh, Pa.) supplemented with 5% fetal calf serum and 2 mM L-glutamine (GIBCO BRL, Grand Island, N.Y.). These cells were used between passages 10 and 15 in culture. Human dermal microvascular endothelial cells were purchased from Clonetics (San Diego, Calif.). Cells were grown to confluence in endothelial cell basal medium-2 (Clonetics) containing 2% fetal calf serum and the cell growth supplements provided by the company. These cells were used between passages 5 and 10 in culture. Based on the work of numerous investigators who have demonstrated that endothelial cells from different sources have similar properties, we hypothesized that the adrenal capillary and dermal microvascular endothelial cells used in this study were competent to respond, in similar ways, to stimuli such as hypoxia and VEGF.[32–36] Moreover, on the basis of the literature, it is plausible that the adrenal capillary and dermal microvascular endothelial cell response (i.e., BMP-2 production) to these stimuli is representative of the endothelial cell response in a fracture microenvironment. All of the following experiments were performed in duplicate. The experiments are reproducible and the figures are representative of our findings.

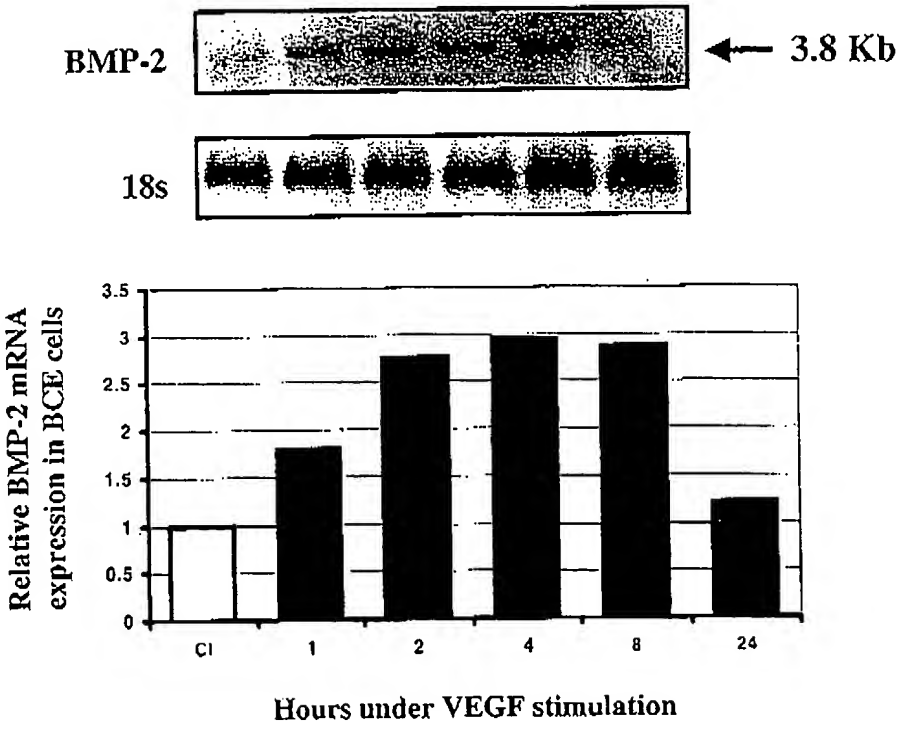

Fig. 2. The effects of rhVEGF stimulation on BMP-2 mRNA expression in bovine capillary endothelial cells in vitro. Confluent bovine capillary endothelial cell cultures were treated with rhVEGF (25 ng/ml) for 1, 2, 4, 8, or 24 hours and were compared with unstimulated cell cultures (*Cl*, control). A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression at the indicated time points. The intensity of BMP-2 hybridization is given as a value relative to unstimulated bovine capillary endothelial cells. rhVEGF stimulation resulted in a 3-fold increase in BMP-2 mRNA expression, peaking after 4 hours of exposure to rhVEGF. BMP-2 mRNA expression then returned to baseline level after 24 hours of treatment. This experiment was performed in duplicate.

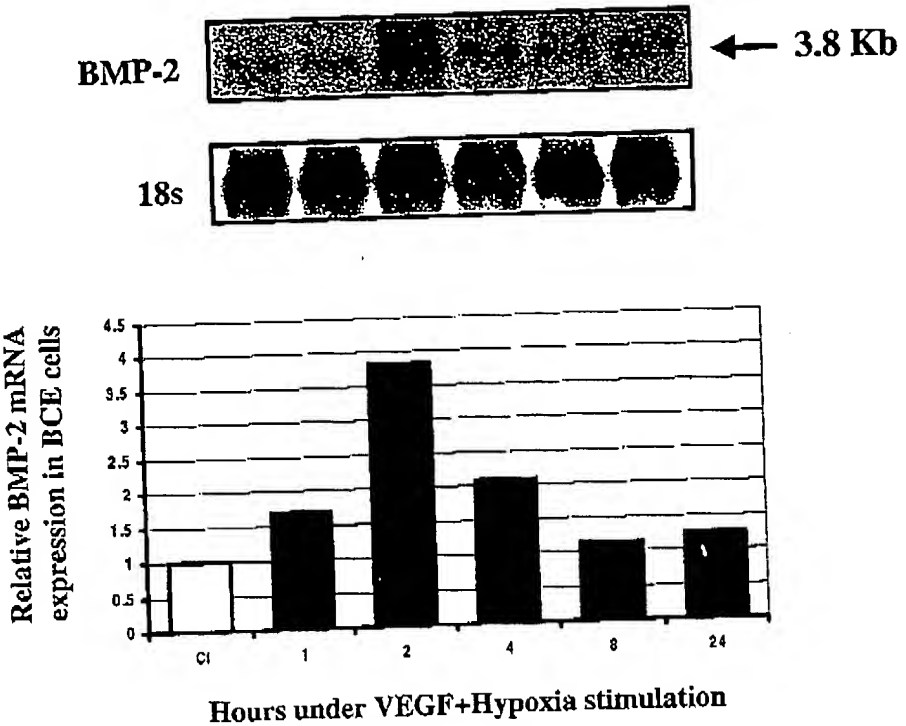

FIG. 3. The combined effects of rhVEGF and hypoxia on BMP-2 mRNA expression in bovine capillary endothelial cells in vitro. Confluent bovine capillary endothelial cell cultures were treated with rhVEGF (25 ng/ml); immediately placed in hypoxic conditions ($Po_2 < 1\%$) for 1, 2, 4, 8, or 24 hours; and compared with unstimulated cell cultures (*Cl*, control). A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression at the indicated time points. The intensity of BMP-2 hybridization is given as a value relative to unstimulated bovine capillary endothelial cells. Costimulation with rhVEGF and hypoxia resulted in a 4-fold increase in BMP-2 mRNA expression, peaking at 2 hours of stimulation and returning near baseline level by 24 hours of treatment. This experiment was performed in duplicate.

Experimental Culture Conditions

Hypoxia stimulation. Hypoxia experiments were performed in both cell types using a standard Plexiglas chamber (Bellco Glass, Vineland, N.J.) deoxygenated by positive infusion of a 5% carbon dioxide/95% nitrogen gas mixture. Cells were starved for 12 hours in serum-free medium and then placed in the hypoxia chamber. Equal atmospheric pressure was ensured by monitoring infusion with a standardized pressure gauge. During the experiment, cell cultures were placed in a standard humidified tissue incubator at 37°C and continuous oxygen saturation was monitored and kept below 1%. Total cellular RNA was then isolated after 0, 1, 2, 4, 8, and 24 hours.

VEGF treatment. Recombinant human VEGF (rhVEGF) was purchased from Intergen (Purchase, N.Y.). Confluent bovine capillary endothelial and human microvascular endothelial cell cultures were starved for 12 hours in serum-free medium. Cultures were then treated with 25 ng/ml of rhVEGF, on the basis of a previous dose-response experiment showing peak BMP-2 mRNA expression with 25 ng/ml of rhVEGF (data not shown). Total cellular RNA was isolated after 0, 1, 2, 4, 8, and 24 hours.

Hypoxia and VEGF. To determine whether there was a synergistic or additive effect between hypoxia and VEGF, bovine capillary endothelial and human microvascular endothelial cell cultures were starved for 12 hours in serum-free medium, and then exposed to 25 ng/ml of rhVEGF in combination with hypoxia. Total cellular RNA was then isolated after 0, 1, 2, 4, 8, and 24 hours.

Actinomycin D and cycloheximide pretreatment. To investigate the mechanisms of hypoxia-induced BMP-2 mRNA up-regulation, confluent bovine capillary endothelial cell cultures were treated with actinomycin D (5 μg/ml), an inhibitor of RNA transcription, 3 hours

before placement in the hypoxia chamber. Total cellular RNA was isolated 2 hours after hypoxic stimulation, and Northern blot analysis was performed as described below. To determine whether hypoxia-induced BMP-2 mRNA up-regulation required de novo protein synthesis, confluent bovine capillary endothelial cell cultures were treated with cycloheximide (10 μg/ml), an inhibitor of protein translation, 2 hours before stimulation with hypoxia. Total cellular RNA was isolated 2 hours after hypoxic stimulation, and Northern blot analysis was performed as described below. All experiments were performed in duplicate.

To determine whether hypoxia affected BMP-2 mRNA stability, confluent bovine capillary endothelial cell cultures were incubated in hypoxia or normoxia. Two hours later, we added 5 μg/ml of actinomycin D. Total cellular RNA was isolated just before the addition of actinomycin D and then 2, 4, and 6 hours after its addition. BMP-2 Northern blot analysis was then performed as described below, and relative amounts of BMP-2 mRNA were expressed as percentages of the value at time 0 (just before the addition of actinomycin D). It is important to note that because baseline BMP-2 expression in unperturbed endothelial cells was low, the gain on the PhosphoImager and ImageQuant software was uniformly increased to facilitate precise detection of baseline BMP-2 signal and its degradation (see Northern Blot Analysis section for PhosphoImager and ImageQuant details). This experiment was performed in duplicate.

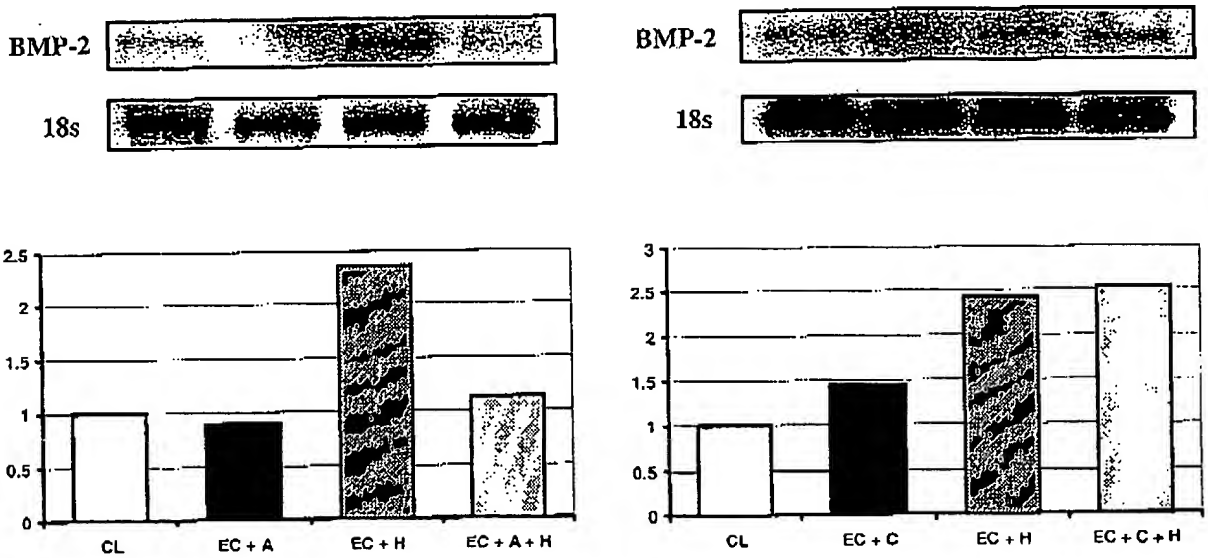

FIG. 4. (*Left*) The effects of actinomycin D pretreatment on BMP-2 mRNA expression in hypoxic bovine capillary endothelial cells in vitro. Confluent bovine capillary endothelial cell cultures (*EC*) were treated with 5 μg/ml actinomycin D (*A*) for 3 hours and then placed in hypoxic (*H*) conditions for 2 hours. A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression in the different experiments: *CL*, control; *EC* + *A*, bovine capillary endothelial cells pretreated with 5 μg/ml actinomycin D for 3 hours; *EC* + *H*, bovine capillary endothelial cells placed in hypoxia for 2 hours; *EC* + *A* + *H*, bovine capillary endothelial cells pretreated with 5 μg/ml actinomycin D for 3 hours followed by hypoxia stimulation for 2 hours. The intensity of BMP-2 hybridization is given as a value relative to unstimulated bovine capillary endothelial cells. Hypoxia stimulation resulted in a 2.4-fold increase in BMP-2 mRNA expression in control cultures. In contrast, pretreatment with actinomycin D, an inhibitor of RNA transcription, prevented BMP-2 mRNA up-regulation as compared with hypoxic stimulation alone, suggesting that hypoxic induction of BMP-2 mRNA is a transcriptionally mediated event. This experiment was performed in duplicate. (*Right*) The effects of cycloheximide pretreatment on BMP-2 mRNA expression in hypoxic bovine capillary endothelial cells in vitro. Confluent bovine capillary endothelial cell cultures (*EC*) were treated with 10 μg/ml cycloheximide (*C*) for 2 hours and then placed in hypoxic (*H*) conditions for 2 hours. A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression in the different experiments: *CL*, control, *EC* + *C*, bovine capillary endothelial cells pretreated with 10 μg/ml cycloheximide for 2 hours; *EC* + *H*, bovine capillary endothelial cells placed in hypoxia for 2 hours; *EC* + *C* + *H*, bovine capillary endothelial cells pretreated with 10 μg/ml cycloheximide for 2 hours followed by hypoxia stimulation for 2 hours. The intensity of BMP-2 hybridization is given as a value relative to unstimulated bovine capillary endothelial cells. In contrast to pretreatment with actinomycin D (*left*), the addition of cycloheximide to the hypoxia-stimulated cultures did not suppress the up-regulation of BMP-2 mRNA compared with nontreated controls. These experiments suggest that de novo protein synthesis is not required for the transcriptional BMP-2 mRNA up-regulation in bovine capillary endothelial cells by hypoxia. This experiment was performed in duplicate

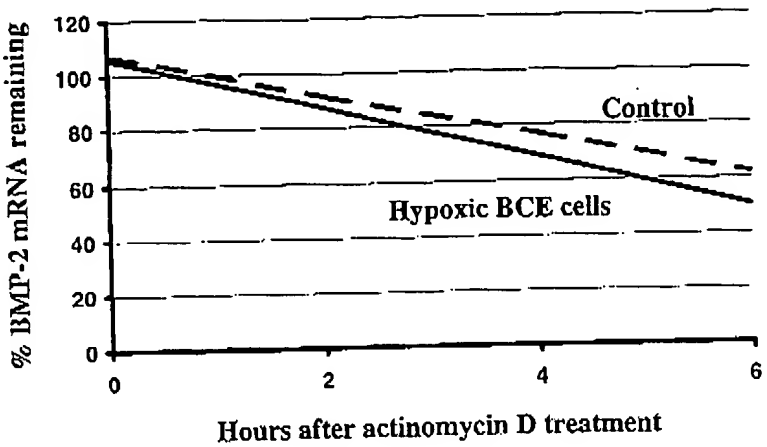

FIG. 5. The effects of hypoxia on bovine capillary endothelial cell BMP-2 mRNA stability in vitro. Confluent bovine capillary endothelial cell cultures were incubated in hypoxia or normoxia for 2 hours and then treated with actinomycin D (5 μg/ml). Total cellular RNA was isolated just before and then 2, 4, and 6 hours after the addition of actinomycin D, and the rate of BMP-2 mRNA degradation was determined as a function of time. Regression analysis suggests that the slopes of BMP-2 mRNA degradation curves are similar between the hypoxic (*solid line*) and the normoxic (*dashed line*) bovine capillary endothelial cells. This suggests that hypoxia increases BMP-2 mRNA expression through transcriptional mechanisms and not through an increased mRNA stability. It is important to note that because baseline BMP-2 expression in unperturbed endothelial cells was low, the gain on the PhosphoImager and ImageQuant software was uniformly increased to facilitate precise detection of baseline BMP-2 signal and its degradation. This experiment was performed in duplicate.

Northern Blot Analysis

The cDNA for rat BMP-2 was a generous gift of Dr. S. E. Harris (San Antonio, Texas).[37] The rat cDNA probe for 18s ribosomal RNA was a 334-bp polymerase chain reaction–amplified segment cloned into the EcoRI site of a pCR2.1 vector.[38] The 18s probe was sequenced to confirm its identity. One hundred nanograms of each probe were labeled with 50 μCi of (α^{32}P)d-CTP (New England Nuclear Life Sciences, Boston, Mass.) using the random primer technique (Pharmacia Biotech, Piscataway, N.J.). Probes were purified from unlabeled nucleotides using Sephadex G-50 DNA grade NICK columns (Pharmacia Biotech). A specific activity of at least 1×10^5 cpm/ml of hybridization solution was used for all experiments.

Northern blot analysis was performed as previously described.[39] Briefly, cells were washed with phosphate-buffered saline and lysed with TRIzol reagent (Life Technologies, Grand Island, N.Y.), and total cellular RNA was extracted and quantified using a spectrophotometer (Molecular Dynamics, Sunnyvale, Calif.). Twenty micrograms of total cellular RNA was fractionated on a 1% formaldehyde denaturing gel, transferred to a Nytran positively charged nylon membrane (Schleicher & Scheull, Keen, N.H.), and cross-linked by ultraviolet light (Stratagene, La Jolla, Calif.). Membranes were prehybridized with ExpressHyb solution (Clontech, Palo Alto, Calif.) at 68°C for 1 hour, followed by hybridization with the (α^{32}P)d-CTP–labeled BMP-2 cDNA probe for 4 hours at 68°C. Stringency washes were performed with 1× sodium saline citrate and 0.1% sodium dodecyl sulfate at room temperature for 10 minutes followed by 0.1× sodium saline citrate and 0.1% sodium dodecyl sulfate at 50°C for 10 minutes. Membranes were exposed to phosphor screens (Molecular Dynamics) for 12 hours. Densitometry of phosphor screens was performed using a PhosphorImager (Molecular Dynamics), and images were quantified using the ImageQuant image analysis software program (Molecular Dynamics). Equal RNA loading and uniformity of blot transfer were assessed by stripping and reprobing the same membranes with a probe for 18s rRNA. All experiments were performed in duplicate.

Western Blot Analysis

Confluent bovine capillary endothelial cultures grown in 10-cm plates were fed with 4 ml of serum-free media. Supernatants were collected after 0, 24, and 48 hours of treatment with hypoxia or rhVEGF. Because BMP-2 is heparin binding, 1 ml of supernatant was

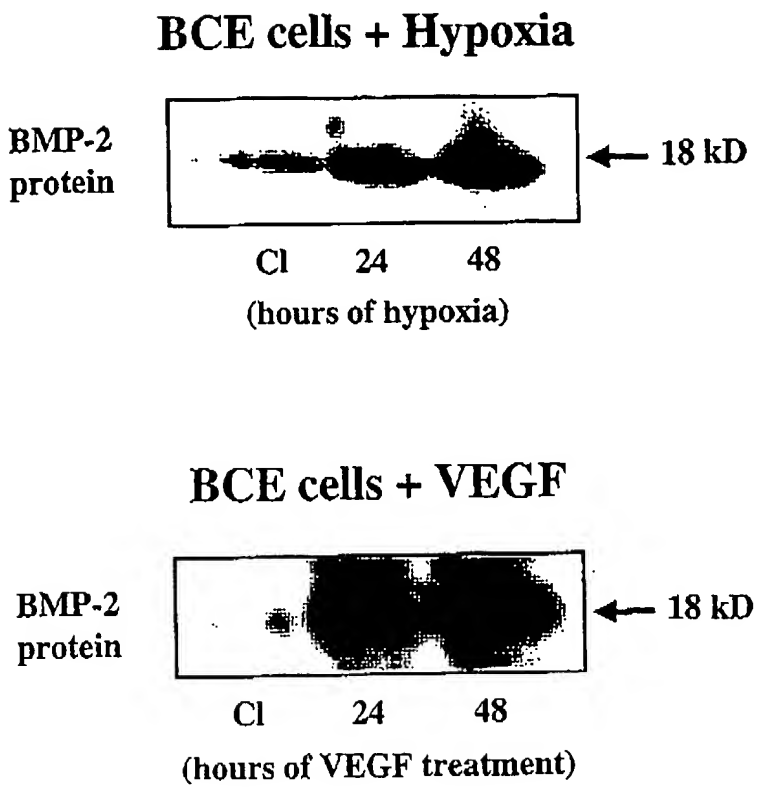

FIG. 6. The effects of hypoxia or rhVEGF stimulation on BMP-2 protein expression in bovine capillary endothelial cells in vitro. BMP-2 protein expression was analyzed in bovine capillary endothelial cell culture supernatants just before hypoxia or rhVEGF treatment, and after 24 and 48 hours of stimulation. Supernatants were harvested and Western blot analysis for BMP-2 was performed using a polyclonal antibody against human BMP-2. We observed a marked increase in BMP-2 protein expression after 24 and 48 hours of treatment with either hypoxia (*above*) or rhVEGF (*below*) as compared with unstimulated cells. This experiment was performed in duplicate.

mixed with Immobilized Heparin Beads Gel (Pierce Chemical Co., Rockford, Ill.) for 1 hour. The beads were pelleted and the supernatant was discarded. After heating to 95°C to dissociate beads and heparin-bound BMP-2 proteins, the samples were fractionated on a 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, Mass.). Western blot analysis for BMP-2 was performed using a polyclonal antibody against human BMP-2 (R&D Systems, Minneapolis, Minn.). This antibody had strong cross-reactivity with bovine BMP-2. Immunoreactivity was determined using the ECL chemiluminescence detection system (Amersham, Arlington Heights, Ill.). This experiment was performed in duplicate.

Crystal Violet Staining

To standardize BMP-2 protein production for cell number, crystal violet staining of the culture dishes was performed using a modification of the methods of Kueng et al.[40] Briefly, cells were washed with phosphate-buffered saline and fixed in ice-cold 3.7% paraformaldehyde (Sigma Chemical Co., St. Louis, Mo.) for 20 minutes. Cells were then washed with phosphate-buffered saline, permeabilized with 20% methanol for 20 minutes, and stained with 0.5% crystal violet (Sigma) in 20% methanol for 30 minutes. Cells were destained by three washes in deionized water, followed by elution of the residual crystal violet with 10% acetic acid for 30 minutes. Optical density was measured by spectrophotometry at 650 nm (Pharmacia Biotech, Cambridge, United Kingdom). The optical density obtained with this assay has been shown to correlate directly with cell number and is thus useful for standardization of cell number between experimental cultures. This experiment was performed in duplicate.

RESULTS

BMP-2 mRNA Expression Is Increased by Hypoxia in a Time-Dependent Manner in Bovine Capillary Endothelial Cells

To assess the effects of hypoxia on BMP-2 mRNA expression, bovine capillary endothelial cells were exposed to hypoxia ($Po_2 < 1\%$) for 0, 1, 2, 4, 8, and 24 hours. BMP-2 mRNA levels were compared with cells maintained at normoxia. Experiments were performed in duplicate. For all experiments, we observed a transcript size of approximately 3.8 kb, as expected.[37] These results are summarized in Figure 1. Hypoxic conditions resulted in a 2.5-fold increase in BMP-2 mRNA expression after 2 hours. BMP-2 mRNA expression then gradually returned almost to the baseline level.

BMP-2 mRNA Expression Is Increased by rhVEGF Stimulation in a Time-Dependent Manner in Bovine Capillary Endothelial Cells

To assess the effects of rhVEGF stimulation on BMP-2 mRNA expression, confluent bovine capillary endothelial cell cultures were stimulated with 25 ng/ml of rhVEGF for 1, 2, 4, 8, or 24 hours and compared with unstimulated cells. Experiments were performed in duplicate. These results are summarized in Figure 2. rhVEGF stimulation resulted in a 3-fold increase in BMP-2 mRNA expression, peaking after 4 hours of exposure to rhVEGF. BMP-2 mRNA expression returned to baseline level after 24 hours of treatment.

Combined rhVEGF plus Hypoxia Stimulation Induces a Marked Increase in BMP-2 mRNA Expression in Bovine Capillary Endothelial Cells

To investigate the combined effects of rhVEGF and hypoxia on BMP-2 mRNA expression in bovine capillary endothelial cells, confluent cultures were stimulated with 25 ng/ml of rhVEGF and then immediately placed in hypoxic conditions ($Po_2 < 1\%$) for 1, 2, 4, 8, or 24 hours. BMP-2 mRNA levels were compared with unstimulated cells. Experiments were performed in duplicate. These results are summa-

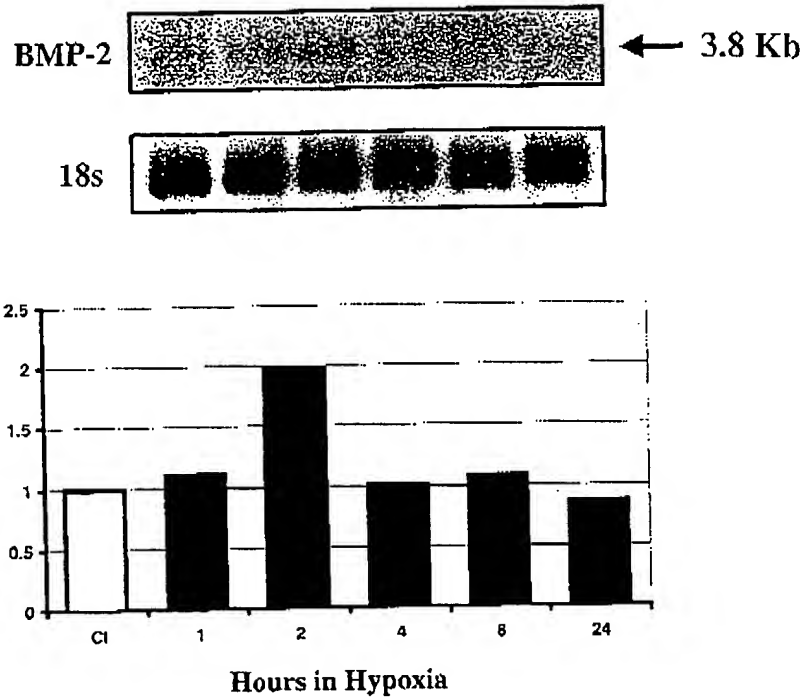

FIG. 7. The effects of hypoxia on BMP-2 mRNA expression in human microvascular endothelial cells in vitro. Confluent human microvascular endothelial cell cultures were placed in hypoxia for 1, 2, 4, 8, or 24 hours or normoxia (*Cl*, control). A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression at the indicated time points. The intensity of BMP-2 hybridization is given as a value relative to unstimulated human microvascular endothelial cells. Hypoxic conditions resulted in a 2-fold increase in human microvascular endothelial cell BMP-2 mRNA expression approximately 2 hours after exposure to hypoxia. This was similar to the findings in bovine capillary endothelial cells (Fig. 1). This experiment was performed in duplicate.

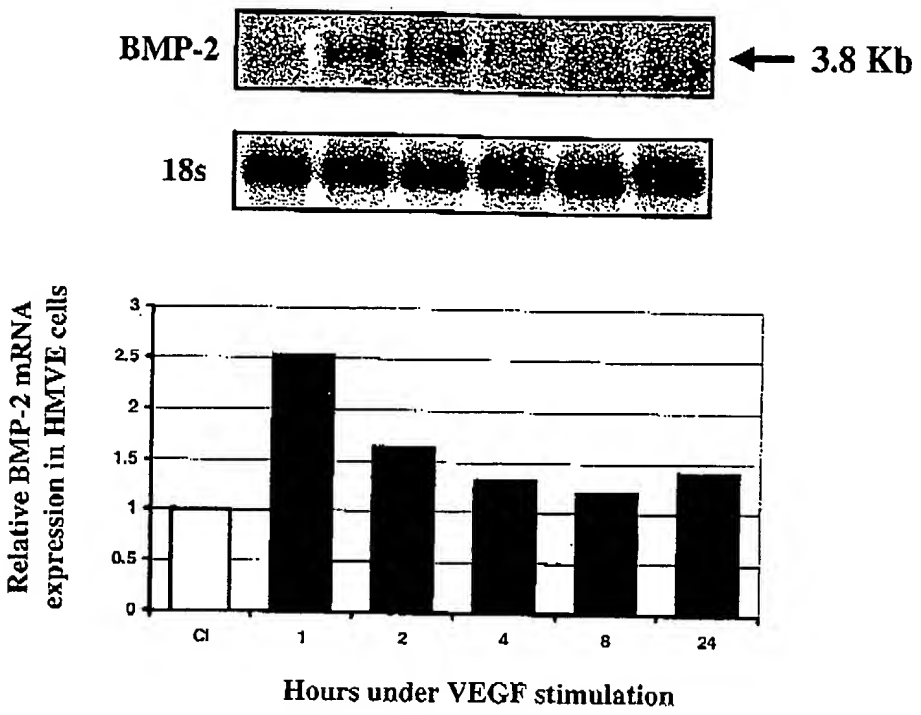

FIG. 8. The effects of rhVEGF stimulation on BMP-2 mRNA expression in human microvascular endothelial cells in vitro. Confluent human microvascular endothelial cell cultures were treated with rhVEGF (25 ng/ml) for 1, 2, 4, 8, or 24 hours and were compared with unstimulated cell cultures (*Cl*, control). A representative Northern blot with 18s control is shown above. The graph shows relative levels of BMP-2 mRNA expression at the indicated time points. The intensity of BMP-2 hybridization is given as a value relative to unstimulated human microvascular endothelial cells. rhVEGF stimulation resulted in a 2.5-fold increase in human microvascular endothelial cell BMP-2 mRNA expression after 1 hour of treatment. This finding was similar to bovine capillary endothelial cells (Fig. 2) except human microvascular endothelial cells up-regulate BMP-2 expression more rapidly. This experiment was performed in duplicate.

rized in Figure 3. Costimulation with rhVEGF and hypoxia resulted in a 4-fold increase in BMP-2 mRNA expression, peaking at 2 hours of stimulation and returning to baseline level after 24 hours of treatment. Although hypoxia and rhVEGF appeared to have an additive effect on BMP-2 mRNA expression in bovine capillary endothelial cells, this effect was slightly less than the predicted value (4-fold versus 5.5-fold).

Hypoxia-Induced BMP-2 mRNA Up-Regulation Is Transcriptionally Regulated and Not Dependent on De Novo Protein Synthesis in Bovine Capillary Endothelial Cells

To determine whether the BMP-2 mRNA up-regulation was related to an increased transcriptional rate, confluent bovine capillary endothelial cell cultures were pretreated with actinomycin D and then placed in hypoxic conditions as previously described. Experiments were performed in duplicate. Hypoxia stimulation resulted in a 2.4-fold increase in BMP-2 mRNA expression in control cultures. This was almost identical to results observed in Figure 1. In contrast, pretreatment with actinomycin D, an inhibitor of RNA transcription, prevented BMP-2 mRNA up-regulation, suggesting that hypoxia induction of BMP-2 mRNA is a transcriptionally mediated event (Fig. 4, *left*).

To ascertain whether the transcriptional increase in BMP-2 mRNA in response to hypoxia required de novo protein synthesis, bovine capillary endothelial cells were pretreated with cycloheximide and then placed in hypoxic conditions as previously described. Experiments were performed in duplicate. In contrast to pretreatment with actinomycin D, the addition of cycloheximide to the hypoxia-stimulated cultures did not suppress the up-regulation of BMP-2 mRNA compared with nontreated controls (Fig. 4, *right*). These experiments suggest that de novo protein synthesis is not required for the transcriptional BMP-2 mRNA up-regulation.

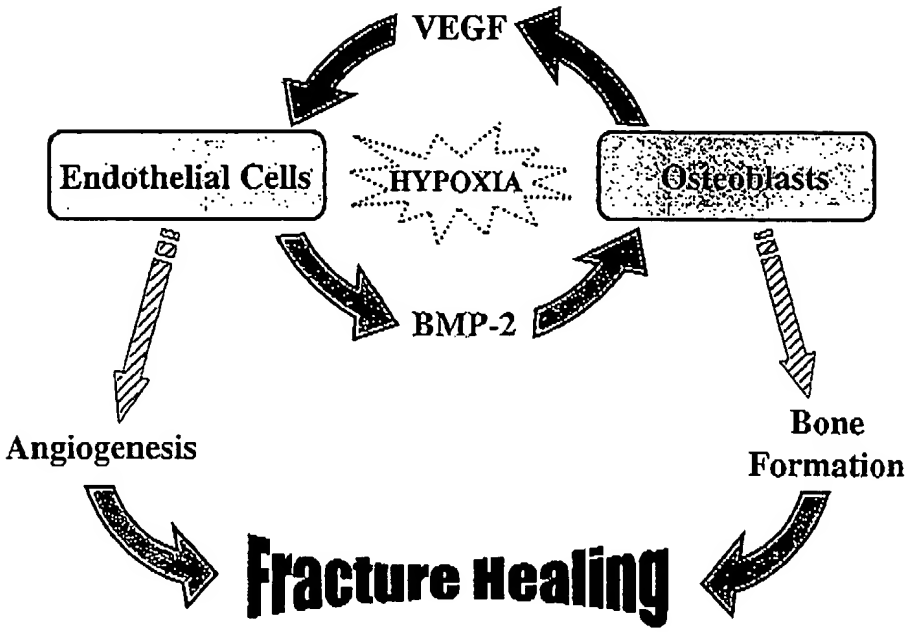

FIG. 9. Schematic representation of the relationships between endothelial cells and osteoblasts at the fracture site. The results of our study suggest that endothelial cells may be involved in the fracture repair process through the expression of BMP-2. Endothelial cells respond to fracture site stimuli such as hypoxia or VEGF and in turn produce BMP-2 that can potentially act back on osteoblasts as an osteogenic signal. Thus, endothelial cells could play a role in the fracture healing process not only by reconstructing the disrupted vasculature but also by directly stimulating osteoblasts through the enhanced expression of a potent osteogenic factor such as BMP-2.

BMP-2 mRNA Stability Is Unaffected by Hypoxia in Bovine Capillary Endothelial Cells

Because the increase in BMP-2 transcript levels could be attributable to an increased transcription rate or an increased mRNA stability, we examined the effect of hypoxia on BMP-2 mRNA stability. Confluent bovine capillary endothelial cell cultures were incubated in hypoxia or normoxia for 2 hours and then treated with actinomycin D (5 μg/ml). Total cellular RNA was isolated just before and then 2, 4, and 6 hours after the addition of actinomycin D, and the rate of BMP-2 mRNA degradation was determined as a function of time. Experiments were performed in duplicate. Relative amounts of BMP-2 mRNA were expressed as percentages of the value at time 0 (just before the introduction of actinomycin D). The results of this study are summarized in Figure 5. In these experiments, no marked difference was noted between the slopes of the regression analysis curves of the hypoxia-treated and untreated bovine capillary endothelial cells BMP-2 mRNA curves. These results suggest that BMP-2 mRNA stability was not affected by hypoxia.

BMP-2 Protein Expression Is Increased by Hypoxia or rhVEGF Treatment in Bovine Capillary Endothelial Cells

Next, we analyzed BMP-2 protein expression in response to hypoxia or rhVEGF stimulation. Experiments were performed in duplicate. The results are summarized in Figure 6. BMP-2 protein expression was standardized for cell number using a crystal violet staining assay, as described in the Materials and Methods section. We observed a marked increase in BMP-2 protein expression after 24 and 48 hours of treatment with either hypoxia or rhVEGF as compared with unstimulated cells.

BMP-2 mRNA Expression Is Up-Regulated in Hypoxia or rhVEGF-Stimulated Human Microvascular Endothelial Cells

To determine whether the hypoxia- and rhVEGF-induced up-regulation in BMP-2 mRNA expression was restricted to one specific endothelial cell line, we applied similar hypoxic or VEGF stimulation to human microvascular endothelial cells. Experiments were performed in duplicate. Hypoxic conditions resulted in a

2-fold increase in human microvascular endothelial mRNA expression of BMP-2 at approximately 2 hours after exposure to hypoxia (Fig. 7). rhVEGF stimulation resulted in a 2.5-fold increase in human microvascular endothelial cell BMP-2 mRNA expression after 1 hour of treatment (Fig. 8).

Discussion

Recent evidence suggests that bone fracture healing involves the interaction of numerous cell types (e.g., osteoblasts, endothelial cells, fibroblasts), cytokines (e.g., bone morphogenetic proteins, FGF-2, TGF-β1), and environmental factors (e.g., hypoxia, pH) at the fracture site.[6,39,41–43] Recent studies have highlighted the fact that the role of some cells involved in the fracture repair process may be much more complex than initially thought. Osteoblasts, for example, primarily considered to be a simple and efficient "osteoid matrix factory," have recently been shown to produce VEGF, a potent angiogenic cytokine.[25,27] Thus, it seems that osteoblasts not only play an osteogenic role in fracture repair but also facilitate bone repair by stimulating angiogenesis at the fracture site through the expression of VEGF. Moreover, VEGF is now considered to be a key mediator in the angiogenic process required during bone repair and remodeling.[24,30] We were surprised by the data presented herein that suggested endothelial cells could play not only an angiogenic role but also potentially an osteogenic role by directly stimulating osteoblasts, through the enhanced expression of a potent osteogenic factor, BMP-2.

The relationship between endothelial cells and the bone morphogenetic protein family is not new. BMP-2 was first shown to be present in the vasculature by Bostrom et al., who observed BMP-2 mRNA in the atherosclerotic plaques of human arteries.[44] BMP-6 mRNA was also observed in atherosclerotic lesions by Schluesener and Meyermann.[45] Li et al. recently reported the presence of BMP-2 mRNA in bone marrow endothelial cells.[46] Thus, BMPs are now considered to be ubiquitous in the vasculature; yet, the functional significance of their presence remains unclear and controversial. It has been suggested by Willette et al. that BMP-2 is mitogenic for vascular smooth muscle cells and could play a role in the structural remodeling accompanying chronic stresses of the vasculature.[47] However, Nakaoka et al. reported a decrease in the proliferation of rat vascular smooth muscle cells treated with BMP-2.[48] How can endothelium-derived BMP-2 have different effects in different tissues? Although first identified for its capacity to promote ectopic bone formation, BMP-2 may regulate a myriad of cellular processes, including differentiation, proliferation, apoptosis, adhesion, and migration.[21,49,50] Diffusion of active BMP-2 establishes a concentration gradient that guides cellular response by initiating a cascade of gene expression.[51] Interestingly, because extracellular factors (e.g., bone morphogenetic protein antagonists) can shape BMP-2 morphogenetic gradients, the local microenvironment (e.g., adrenal gland, dermis, or fracture site), more so than the individual type of endothelial cells, may be responsible for contouring the effects of BMP-2.[20] In other words, BMP-2 secreted by endothelial cells in the adrenal gland or dermis may have very different effects than BMP-2 secreted by endothelial cells present at a fracture site; however, this hypothesis remains to be proven.

The results of the present study suggest that hypoxia or rhVEGF stimulation induced an upregulation of BMP-2 mRNA and protein expression in two different types of microvascular endothelial cells (bovine capillary endothelial and human microvascular endothelial cells). However, it is now established that VEGF itself is induced by low oxygen tension in a variety of cell types, including endothelial cells and osteoblasts.[8,25,52] Because hypoxia-induced VEGF upregulation is controlled at both the transcriptional and posttranscriptional levels,[23,53] it could be argued that the hypoxia-induced BMP-2 upregulation observed in our experiments was mediated by a hypoxia-induced VEGF mRNA upregulation. However, pretreatment of the cell cultures with cycloheximide, to block de novo VEGF protein synthesis, did not impair BMP-2 mRNA expression in bovine capillary endothelial cells. Thus, it is likely that hypoxia directly upregulates the transcription of BMP-2 mRNA in the microvascular endothelial cells studied. Interestingly, the combined effects of hypoxia and rhVEGF resulted in an additive increase in BMP-2 mRNA expression in bovine capillary endothelial cells. Future studies are needed to determine whether hypoxia and VEGF share common or independent intracellular signaling pathways.

Hypoxia has long been known to up-regulate gene expression and secretion of some potent mitogenic factors such as PDGF-BB, TGF-β1, and thrombospondin-1 in endothelial cells.[4,9,10,13] FGF-2 expression has also been

shown to be enhanced through the release of endocellular storage, as its synthesis is not augmented by hypoxic conditions.[10] These growth factors (and others such as interleukin-1, interleukin-6, and colony-stimulating factors) are known to control the recruitment, proliferation, differentiation, and function of various cells, including osteoblasts and osteoclasts.[43] Interestingly, in a parallel series of hypoxia experiments, primary osteoblastic cells (derived from neonatal rat calvarial osteoblasts) did not up-regulate BMP-2 mRNA (data not shown). This may suggest, in the early fracture microenvironment, that endothelial cells are the primary sources of BMP-2.

Hypoxia-induced up-regulation of cytokines is considered to be a carefully regulated physiologic event, mediated by several second messengers, protein kinases, and transcription factors.[9,54] Heme-containing proteins serve as a dedicated oxygen sensor in endothelial cells, generating a signal by altering the redox potential of the cell.[55] Moreover, transcription factors such as activating protein-1 (AP-1), nuclear factor-κB (NF-κB), and hypoxia-inducible factor-1 (HIF-1) are (reversibly) activated in endothelial cells as a function of the oxygen tension.[56–58] However, the mechanism of regulation differs from one hypoxia-induced cytokine to another. For example, PDGF-BB is regulated at the transcriptional level, thrombospondin-1 is regulated at the posttranscriptional level, and VEGF is regulated at both the transcriptional and posttranscriptional levels.[13,52,59] In this study, we observed that the hypoxia-induced BMP-2 mRNA up-regulation was controlled at the transcriptional level in bovine capillary endothelial cells. Indeed, pretreatment of confluent bovine capillary endothelial cell cultures with actinomycin D prevented the hypoxia-induced BMP-2 mRNA up-regulation. Accordingly, we observed similar rates in mRNA degradation between endogenous and hypoxia-induced BMP-2 mRNA, suggesting that hypoxia does not affect mRNA stability, but instead regulates BMP-2 mRNA expression primarily at the transcriptional level. Finally, the rapid endothelial cell response to hypoxia observed in this study suggests that an early transcription factor (e.g., HIF) could mediate the hypoxia-induced up-regulation of BMP-2 mRNA expression in endothelial cells. However, further evidence is needed to support this hypothesis.

In summary, it seems that endothelial cells are members of a complex communication network that involves endothelial cells, osteoblasts, and probably other cell types as well.[43] Considering the intimate spatial relationship between endothelial cells and osteoblasts at the fracture site, some authors have suggested that the endothelial cell itself could be an osteoblast progenitor cell.[1,41,42,60,61] Moreover, several studies have shown that there are reciprocal regulatory and functional relationships between endothelial cells and osteoblasts during osteogenesis. For example, Jones et al. have demonstrated that microvascular endothelial cells can increase osteoblast proliferation.[62] Moreover, endothelial cells have been shown to increase osteoblast proliferation without increasing osteoblast collagen synthesis.[63] The in vivo studies of Villanueva and Nimni and Decker et al. also suggest that endothelial cells increase bone formation and extracellular matrix mineralization along with an increase in neovascularization.[41,61,64] Although Guenther et al. reported that endothelial cells in culture were able to synthesize a "potent bone cell active mitogen," the mechanisms of these close interrelations remain unclear.[63] We have previously shown that hypoxia increases VEGF expression in osteoblasts.[25,26] Here, surprisingly, we demonstrate that hypoxia also increases BMP-2 expression in endothelial cells. The results of our in vitro study suggest that endothelial cells may be involved in an in vivo reciprocal fracture repair loop. Endothelial cells may respond to fracture site stimuli such as hypoxia or VEGF and, in turn, produce BMP-2 that can act reciprocally on osteoblasts as an osteogenic signal (Fig. 9).

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